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Epithelin/granulin precursor is an 88 kDa glycoprotein purified as a growth factor called PC cell derived growth factor PCDGF from the culture medium from a highly tumorigenic teratoma-derived cell line called PC. PCDGF belongs to a novel family of double cysteine rich polypeptides including the 6 kDa epithelins and granulins. We show that human mammary carcinoma cell lines MCF7 and MDA-MB-468 express PCDGF mRNA and protein. PCDGF is found both in cell lysate and in culture medium of both cell types indicating that it is being secreted by the mammary carcinoma cells in culture, similarly to what was previously found for the PC cells. The present study is devoted to investigating the hormonal control of PCDGF expression in the mammary carcinoma cell line MCF-7 and to investigating the possible role of PCDGF in the growth of normal and tumorigenic mammary epithelial cells. For this purpose, we will examine the effect of inhibiting PCDGF expression and/or action on the growth of the cells. These experiments will be very important to examine the effect of a novel growth factor on mammary epithelial cell biology.

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G Jones 11/5/97

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First year Progress Report for US Army Grant Proposal DAMD 17-96-1-6072
Epithelin/granulin precursor expression in human breast carcinoma
Ginette Serrero, Ph.D. Principal Investigator.

Epithelin/granulin precursor is an 88 kDa glycoprotein originally purified as an autocrine growth factor from the highly tumorigenic mouse teratoma-derived cell line PC. For this reason this growth factor is defined as PC cell derived growth factor (PCDGF). PCDGF belongs to a novel family of double cysteine rich polypeptides that include the 6 kDa epithelins and granulins originally purified from kidney extracts (epithelins) or granulocyte extracts (granulins). Experiments in the mouse cell lines indicated that PCDGF was overexpressed in the highly tumorigenic cells and that was positively correlated to the tumorigenic properties of the cells.

RATIONALE AND SPECIFIC AIMS

The purpose of the grant proposal DAMD 17-96-1-6072 was to design experiments in order to establish a solid foundation for the role of PCDGF in mammary epithelial cell biology and the involvement of its autocrine expression in the progression of the tumorigenicity in human breast carcinomas.

The specific aims originally proposed were:

1- Comparison of PCDGF expression in normal human mammary epithelial cell strains and in ER+ and ER- breast carcinoma cell lines.
2- Biological activity of PCDGF and its processed form epithelin 1 in normal mammary epithelial cells and mammary carcinoma cells.
3- Effect of inhibition of PCDGF expression (antisense approach) or action (neutralizing antibody or competitive inhibitor approaches) on the growth of malignant breast carcinoma in vitro and in vivo.

PROGRESS REPORT

During the first year of support of the grant application, we have accomplished progress in the three specific aims proposed as described below.
The specific aims rationale and methods used are the same as the ones proposed in the original application and therefore have not been reiterated in the progress report.

1- Comparison of PCDGF expression in normal human mammary epithelial cell strains and in ER+ and ER- breast carcinoma cell lines.

In order to carry out these experiments and in particular in order to measure the expression of PCDGF protein in human cell lines, we had to develop an anti-human PCDGF antibodies since the ones we had developed previously against mouse PCDGF could not adequately recognize human PCDGF by Western blot analysis or by immunoprecipitation.
At present we have succeeded to develop both polyclonal and monoclonal antibodies against human PCDGF using expressed human recombinant PCDGF expressed in E. Coli. These antibodies were used to measure the level of expression of PCDGF protein in human mammary cell lines. Since we have shown that PCDGF was secreted by the cells in their culture medium, we determine the expression of PCDGF in human cell lines both using cell lysates and conditioned media.

Measurement of PCDGF mRNA expression was performed by Northern blot analysis using a human PCDGF cDNA probe.

We examined the level of expression of PCDGF mRNA by Northern blot analysis in the non tumorigenic immortalized breast epithelial cell line MCF10 A, in the estrogen receptor positive cells MCF-7 and in the estrogen receptor negative MDA-MB-468 cells.

The presence of PCDGF protein was examined in MCF7 and MDA-MB-468 cell extracts and also in their culture medium. The method used was a combination of immunoprecipitation with monoclonal anti-PCDGF antibody followed by SDS-PAGE and western blot analysis using polyclonal anti-PCDGF antibody. Using this method, PCDGF was detected both in cell lysates and in culture media of MCF7 and MDA-MB-468 cells respectively. These data indicate that PCDGF is being secreted by human breast carcinoma cells similarly to what was showed previously for the mouse teratoma cell line PC. This is in agreement with the presence of a 17 amino-acid signal peptide in the N-terminus portion of PCDGF (1).

At present time, we are examining the hormonal regulation of PCDGF expression in MCF7 and in MDA 468 cells. The rationale for these studies is the fact that PCDGF is a novel growth factor in breast carcinoma cells and therefore it is of importance to investigate the factors that regulate its expression. PCDGF mRNA expression was measured by northern blot analysis using a human PCDGF cDNA probe. PCDGF protein expression was measured in cell lysates and also in culture medium since PCDGF is a secreted protein by a combination of immunoprecipitation and western blot analysis using anti-human PCDGF antiserum raised in our laboratory.

We showed that PCDGF is expressed in breast cancer cell line MCF-7 which is ER positive and MDA-MB-468 which is ER negative. Both cell types expressed PCDGF mRNA and protein (figure 1). In contrast PCDGF expression in MCF-10A cells which are immortalized non tumorigenic human mammary epithelial cells was very low.

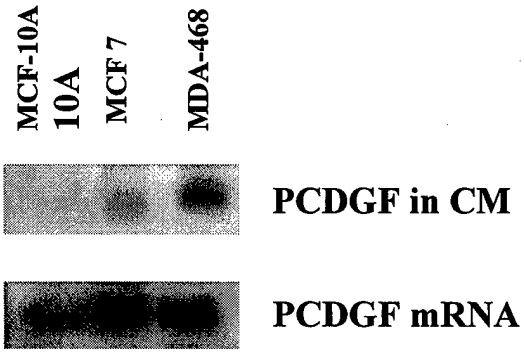


Fig 1: PCDGF expression in human breast cells

These data indicate that PCDGF expression is very low in normal cells and increase with tumorigenic properties in human mammary epithelial cells similarly to what was observed in the mouse model system from which PCDGF was originally isolated.
Based on these results we investigated the effect of estradiol on the expression of PCDGF mRNA and protein in the ER positive cells MCF-7. Our results indicate that 17β-estradiol (E2) stimulates PCDGF expression in a time and dose-dependent fashion (figs 2 and 3)

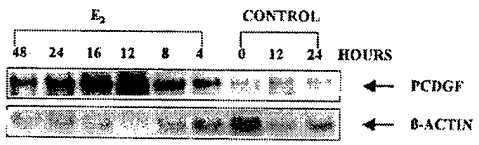


Fig.2: Time dependent increase of PCDGF mRNA expression by E2 (10^{-9} M).

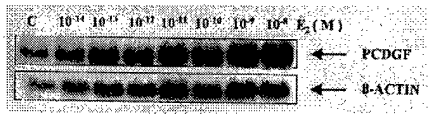


Fig.3: Dose-dependent increase of PCDGF mRNA expression by 12 hr exposure to E2

Northern blot analysis showed that maximal stimulation of PCDGF mRNA expression occurs 12 hours after adding E2 (figure 2) . Dose response studies indicate that maximal stimulation is observed with a concentration of E2 of 10^{-8} M (figure 3) which is the dose known to maximally stimulate TGF-α and IGF-I expression in human breast cancer cells.

Interestingly the stimulatory effect of E2 on PCDGF expression is also prevented by treating the cells with actinomycin D suggesting that the effect is mainly occurring at the transcriptional level and also by the anti-estrogen compound tamoxifen. (fig.4)

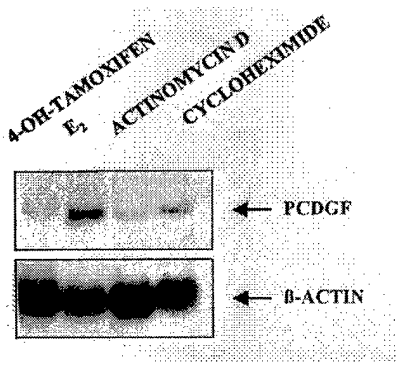


Fig. 4

These results that PCDGF is under estradiol regulation in human mammary epithelial cells are very interesting since they are novel and since they provide information on a novel growth factor which can be a putative candidate growth factor to mediate the growth stimulatory effect of estradiol.
For exploring this hypothesis, it is proposed to develop MCF-7 cells where PCDGF has been inhibited by antisense PCDGF cDNA transfection and examine their growth responsiveness to estradiol. This will be carried out in the present funding period of the grant.

2- Biological activity of PCDGF and its processed form epithelin1 in normal mammary epithelial cells and mammary carcinoma cells.

The properties of PCDGF binding on fibroblastic and epithelial cells were investigated by Scatchard analysis and by affinity labeling of radiolabeled PCDGF. Results indicate that PCDGF cell surface binding sites rea present in cells of various embryonic origin including mammary epithelial cells with an apparent ,molecular weight of 120 kDa (Xia and Serrero, 1998 in press, provided with progress report).
It has been suggested that PCDGF also known as epithelin/granulin precursor contains 7 repeats of 6 kDa. Since 6 kDa epithelins or granulins have been purified from kidney extracts or granulocyte extracts (2-3), it has been suggested that the precursor can be processed into these 6 kDa polypeptide repeats in a biologically controlled processed. It has been proposed to evaluate this possibility in the breast carcinoma cell lines (specific aim 2). However, in order to initiate this investigation, it has been necessary to develop antibodies that would specifically recognize the 6 kDa epithelin processed repeats. For this purpose, we have concentrated our efforts so far into raising such antibodies. These experiments are currently being carried out in our laboratory.

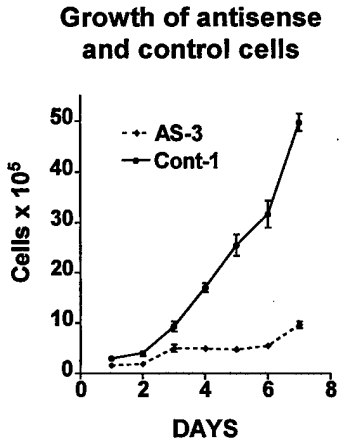
3- Effect of inhibition of PCDGF expression (antisense approach) or action (neutralizing antibody or competitive inhibitor approaches) on the growth of malignant breast carcinoma in vitro and in vivo.

A) Inhibition of PCDGF expression by antisense approach

Studies about the effect of inhibition of PCDGF expression on the tumorigenicity of the cells have initiated. For this purpose, we have constructed an expression vector consisting of pCDNA3 containing a 400 bp fragment of human PCDGF cDNA in the antisense orientation. This antisense cDNA

expression vector was stably transfected into either MDA-MB-468 cells or MCF7 cells. Control cells consisted of corresponding cells transfected with empty vector.

Transfected cells were selected first by their ability to growth in the presence of neomycin since pCDNA3 contains neomycin resistance gene as a selection marker. Neomycin resistant cells were then analyzed for measuring the presence of antisense RNA and for determining if PCDGF expression is efficiently inhibited. Antisense stable transfected cells have been successfully obtained for MCF7 and MDA-MB-468 cells. At present time we are concentrating on investigating the MDA-MB-468 transfected cells because these cells can readily develop into tumors in vivo and therefore tumorigenicity studies should be faster to perform. However, similar studies will also be carried out with antisense and control transfected MCF7 cells.



The comparison of the in vitro growth properties of control and antisense MDA-MB-468 cells are currently being carried out. Our preliminary results indicate that the antisense cells grow at a slower rate that the empty vector control cells (figure 5). We are presently examining whether the growth of the antisense cells can be stimulated by exogenous PCDGF added in the culture medium of the antisense cells.

Experiments are currently carried out to determine the tumorigenic properties of the antisense and control MDA-MD-468 cells in nude mice. For this purpose, efforts are presently spent in expressing and purifying human PCDGF in a biologically active recombinant form.

Fig. 5: Comparison of the growth of empty vector and antisense PCDGF cDNA transfected MDA-468 cells (AS)

B) Inhibition of PCDGF action by using neutralizing antibodies.

These studies are currently going on. For this purpose we have had to develop a neutralizing antibody for human PCDGF. A monoclonal antibody against PCDGF which can neutralize its biological activity has been successfully developed. At present time, purification of the antibody is carried out so that biological studies on its effect on the in vitro and in vivo growth properties of mammary carcinoma cell lines can be carried out.

FUTURE PERSPECTIVES.

The present progress report described here provide some interesting and important results about the expression of PCDGF in human breast carcinoma cells.

The data also show that we have been successful in developing the necessary tools to pursue the specific aims proposed in the original application. Our data show that our proposed studies are feasible and will provide novel information about a novel growth factor expressed in mammary carcinoma and investigate the possible role that these growth factors may play in these cells.

The second year of the grant funding period will be devoted to completing the goals of the specific aims originally proposed in the application as our preliminary results presented here support our working hypothesis of the importance of PCDGF in the growth of mammary carcinoma cells.

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